Original

received 9/2/97

Figs 1-7 inserted 9/2/97

Logged in 11/3/97

replaced p. 8 - 11

HUNTINGTON MEDICAL RESEARCH INSTITUTES

NEUROLOGICAL RESEARCH LABORATORY

734 Fairmount Avenue

Pasadena, California 91105

Contract No. NO1-NS-5-2333

QUARTERLY PROGRESS REPORT

April 1 - June 30, 1997

Report No. 7

"MICROSTIMULATION OF THE LUMBOSACRAL SPINAL CORD"

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This QPR is being sent to you before it has been reviewed by the staff of the Neural Prosthesis Program.

Abstract

In this report we discuss the results from one chronic and four acute experiments. In the chronic study, four microelectrodes implanted in the spinal cord for 21 days were pulsed with our standard chronic stimulation paradigm (12 hrs on each of 2 successive days). This produced no depression of neuronal excitability. The acute experiments were designed to monitor pressure responses within the bladder and entire length of the urethra as the spinal cord was stimulated to elicit sexual reflexes. Stimulation at the S_1 level of the cord produced elevation of the corpus cavernosum pressure and lengthening of the penis. However, while the pressure in penile urethra increased, the stimulation did not induce appreciable changes in the bladder or intraurethral pressure.

We performed comparative histological analysis on three animals in this quarter (SP-74-76). As we pointed out in our previous QPR (No. 6), the electrode arrays failed mechanically in SP-74 and SP-75. Based on necroscopic and histologic analysis, we elaborate on possible explanations including movement of the internal stabilization pad and/or platinum plate (SP-74) and electrical cable separation (SP-76). SP-75 was perfused with a fixative mixture compatible with cytochemical and immunocytochemical analysis of selected spinal cord, brain, and lymphatic tissues. This animal provided valuable tissue material for pilot experiments from which we obtained baseline technical experience. The general histological profile in all three cats indicated that electrode injury produced tissue changes characteristic of a typical stab wound including fibrotic changes with reactive astrocytes and stellate cells associated with thick collagen bundles, especially within the electrode capsule. We also noted inflammatory foci associated with electrodes that penetrated myelinated tissues, possibly implicating an autoimmune-type inflammatory response. Wallerian degenerative changes of the myelin with lipid-laden macrophages was also a major feature within myelinated tissue penetrated by the electrodes. Two general impressions were gleaned from

histological analysis of these three cats: 1) neuronal injury within the vicinity of the electrodes was negligible and 2) the smaller electrode diameter (24 μm) appeared to produce a less severely injured tissue zone compared to that which has been observed in previous experiments employing the larger 50 μm diameter electrodes.

Introduction

The overall goals of this contract are to develop a method of chronic microstimulation of the sacral cord of the cat to effect micturition, and to evaluate the effects of the electrical stimulation on neural and surrounding tissues. In this report we discuss the results from one chronic experiment in which four microelectrodes implanted in the spinal cord for 21 days were stimulated continuously for 12 hrs on each of 2 successive days. Results demonstrated that this prolonged stimulation did not appreciably depress neuronal excitability.

Four acute experiments were conducted aimed at exploring the response of the bladder and urethra to microstimulation of the spinal cord designed to elicit sexual reflexes. Results showed that such stimulation at the S_1 level of the cord produced elevation of the corpus cavernosum pressure and lengthening of the penis. Other than an increase in penile urethral pressure, the stimulation did not induce appreciable changes in the bladder or intraurethral pressure.

Histologic results were obtained from SP-74, -75, and -76. The implanted arrays had failed mechanically proximal to the electrodes. Findings indicated a generalized inflammatory response but suggested that damage near the electrodes was minor. There was also some preliminary evidence that smaller diameter electrodes produce less neural damage.

Methods

Chronic experiments. Adult male cats were anesthetized with 50% nitrous oxide and 1-2% Halothane and the spinal cord exposed as described previously. The S_2 region of the spinal cord was localized by stimulation of the dermatome it serves while recording the dorsal cord potential supradurally, as described previously. Four activated iridium microelectrodes (24-50 μm dia.., 2.8 mm long, 2000 μm^2 exposed stimulating surface) were implanted manually at approximately the

dorsal midline of the spinal cord and angled laterally at about 10 degrees. No matrix was used to support the electrodes. The electrodes were pulsed individually and the bladder lumen pressure was monitored. The electrodes were advanced into the cord until good elevation of bladder pressure was produced by the stimulation. The dura was then closed over the electrodes and the effect of the stimulation again measured. A silastic pad (to which the stimulating electrode leads had been glued) was sutured to the dura to reduce traction on the electrodes. The ground (indifferent) electrode was sutured in place over the microelectrodes. A small hole was made in the dura and a recording electrode inserted so as to lie approximately 3 cm caudal to the stimulating electrodes. A suture was used to secure the recording electrode to the dura. A reference electrode was sutured to the muscle 2 cm above the dura. The wound was flushed with antibacterial solution and the muscle and skin were closed in layers. Subsequent recordings were made with the animal anesthetized with Pentothal (i.v., as needed) or Nembutal (i.v., as needed). A sterile catheter and sterile saline were used during recording of the bladder lumenal pressure.

Acute experiments. Four adult male cat were anesthetized with 50% nitrous oxide and 1-2% Halothane. The spinal cord was exposed using a standard dorsal laminectomy and the rostral extent of the S_2 region was localized in the manner described above. The spinal cord was covered with light mineral oil to prevent drying. The stimulating electrodes were inserted 2-3 mm rostral to the rostral border of the S_2 region using a standard stereotaxic apparatus.

A small pressure transducer (transducer ~1.3 mm dia., catheter ~0.8 mm dia., Millar Instruments) was passed along the length of the urethra and then slowly withdrawn at a constant rate using an electric motor. The frequency response of this pressure transducer is flat to 10 KHz. A potentiometer coupled to the shaft of the motor allowed measurement of the distance the catheter was withdrawn as a function of time. Both the pressure and position signals were digitally stored on tape (18.5 KHz sampling rate per channel). These signals were then digitized off-line at 10 Hz per channel for further

analysis.

Histology. Within 20 minutes of the end of an experiment the animal was anesthetized with Nembutal and perfused through the aorta with saline followed by 2 L of ½ strength Karnovsky's fixative (2% paraformaldehyde and 2.5% glutaraldehyde) in 0.1 M sodium cacodylate buffer, pH 7.4. With the electrodes *in situ*, the complete cord and spinal roots were dissected out to precisely localize the microelectrodes. Two-mm-thick transverse sections containing the electrode tracks were dissected, processed and embedded in epoxy resin. One-μm thick sections were cut serially through the blocks and examined using light microscopy.

Cytochemistry/Immunocytochemistry. Three applications were applied to SP-75 cervical spinal cord tissue samples including: 1) cytochemical detection of the enzyme alkaline phosphatase (AP), an enzyme known to be associated with modulation of blood-brain barrier function, 2) the cytochemical localization of nicotinamide-adenine-dinucleotide phosphate-diaphorase (NADPD-d), which has been suggested to co-localize with NOS in many neuronal populations, and 3) immunocytochemical detection of nitric oxide synthase (NOS), a purported marker of neuronal, glial and other cellular compartments and the enzyme that is responsible for producing nitric oxide (NO), an important neurotransmitter and vasodilator.

This animal was perfused fixed with a weaker fixative that contained 4% formalin, 0.1% glutaraldehyde in a 0.1 M sodium cacodylate buffer with the addition of 0.2 M sucrose at pH:7.4, following blood wash out solution using Ringer's lactate that included heparin 10 units/ml and 0.1% procaine hydrochloride. The fixative recipe is essentially a tissue antigen-friendly fixative and provides adequate structural preservation without destroying antigenic epitopes and membrane-associated enzymes that were targeted for analysis. Such cytochemical and immunocytochemical studies cannot be performed on tissue fixed with the standard ½ strength Karnovsky's fixative primarily because of the high concentration of glutaraldehyde in the latter fixative. Thirty minutes

prior to vascular perfusion, the animal was injected with 3 ml of Ringer's solution containing 286 mg horseradish peroxidase (Sigma Chemical Co., Type II) immediately following intravenous injection of Benydryl to prevent a possible histaminic reaction. Six hours after vascular perfusion, the brain, spinal cord, and lymph nodes were removed and placed into a buffer solution containing 0.1 M sodium cacodylate and 0.2 M sucrose at pH:7.4 (S/C buffer) and held in the refrigerator until use. The sacral spinal cord in which the electrode beads were identified were divided equally into two portions, i.e., one for routine histology and the other for cyto-immunochemistry. Spinal cord samples from the cervical and sacral regions were cut at 50 μm on a Vibratome.

The details of the cytochemical description for alkaline phosphatase will not be presented here. Briefly, the methods used were in accordance with Lossinsky *et al.*, (1983), Mayahara *et al.*, (1967), Wilson and Hodges, (1979) using b-glycerophosphate as the substrate and lead as the capturing agent. In our experience, the best results to demonstrate the sites of AP activity have been obtained using alkaline lead citrate.

Results

Physiologic Results. One chronic (SP-77) and four acute (SP-78 through SP-81) experiments have been completed during this quarter.

In SP-77, a four electrode array was implanted. This array consisted of 34 μm diameter, activated iridium electrodes with straight 25 μm diameter platinum leads. A short (~ 1.5 mm) dural incision was made for each electrode. Each lead was bent into a "U" with padded forceps and the lead inserted through the opening. The electrode was then inserted into the cord leaving about 8-10 mm of lead wire below the dura. After all electrodes had been inserted through their individual openings, fibrin glue was used to seal the top of the dura. The advantage of this procedure is that the incisions are so small that CSF leakage is minimized and the dura/spinal cord does not "deflate." At the end

of the surgery, electrodes 1, 3, and 4 were electrically good (52.5, 50.4, and 54.6 kΩ, respectively) while electrode 2 appeared to be damaged (9.4 kΩ). At 13 days post implant the impedances of the four electrodes were 13.9, 11.0, 49.7, and 24.3 kΩ. At 21 days post implant the impedances of electrodes 1, 3, and 4 were 13.2, 32.1, and 30.68 kΩ while electrode 2 was identified as having a loose connection which may have caused the electrode to appear damaged. At this point, only electrode 4 produced a good evoked potential which had a threshold of about 26 μA. Chronic stimulation was delivered to the spinal cord through electrodes 3 and 4 for 12 hours on each of 2 successive days. The stimulation consisted of balanced charge biphasic, cathodic phase first, 80 μA, 400 μs/ph, 32 nC/ph, 1,600 μC/cm^2, 20 Hz continuous pulses. Figure 1 shows the recruitment curves generated by stimulation of electrode 4 before the start of the first 12 hour stimulation period, just before the start of the second 12 hour stimulation period, and at the end of the second period. Before the start of the second period, neuronal excitability appeared somewhat depressed with the activation threshold increasing to about 30 μA from approximately 10 μA. However, at the end of the second period, the neuronal excitability was virtually identical to that recorded before the first period and, at higher stimulation amplitudes, the excitability was enhanced.

The four acute experiments were aimed at eliciting sexual reflexes via microstimulation of the lumbosacral spinal cord. The best results were obtained in SP-79.

Figure 2 shows baseline activity of the pressure measured within the corpus cavernosum sinus (CCS Pressure, upper trace) and the pressure within the bladder (IU Pressure, lower trace) over a 10 minute period. Spontaneous fluctuations in CCS pressure as large as 10 cmH_2O and typically about 5 cmH_2O occurred at 4-6 minute intervals. Such fluctuations were not correlated with changes in bladder pressure.

Figure 3 shows the response of the CCS pressure and the bladder pressure to stimulation of the S_1 spinal cord with the parameters listed. After stimulation was initiated, there was typically a

delay of 20-50 s before any CCS pressure increase was noted. During such stimulation, the bladder usually showed no response or, at times, a small transient increase in pressure of no more than 10-15 cmH_2O when stimulation was initiated that generally returned to baseline well before any increase in CCS pressure.

Figure 4 demonstrates the intraurethral pressure profile when the CCS pressure was low. Such a profile can be compared to the data from previous QPRs which show the pressure response along the entire length of the urethra as the catheter is withdrawn at a steady rate (1.54 cm/s). The large pressure increase corresponds to the position of the external urethral sphincter (EUS). Note the significant negative pressure just distal to the EUS.

When stimulation elicited a moderate increase in CCS pressure, the intraurethral pressure profile shows three differences in comparison to that elicited without stimulation (Figure 5). First, the large negative pressure is absent. This has been noted in previous studies in which stimulation is applied within the S_2 segment of the cord. Second, the pressure noted within the penile urethra has increased. And, third, the distance from the EUS center to the tip of the penis has increased by 1.08 cm.

When stimulation elicited a large increase in CCS pressure as shown in Figure 6, the intraurethral pressure profile indicates that the EUS pressure has decreased to 80 cmH_2O compared to 90 cmH_2O in the previous two figures. The pressure noted within the penile urethra is correlated with that measured within the CCS. Again, there is no stimulation-induced change in the bladder pressure. The distance from the EUS center to the tip of the penis has increased by 1.69 cm over resting length.

Figure 7 demonstrates the relationship between the CCS pressure and the IU pressure measured 6 mm from the tip of the penis for the data shown in Figures 4 through 6. While the relationship is nonlinear, it is clear that an increase in IU pressure at this point may be a useful

indicator that a particular stimulation paradigm has initiated erection. The value of this lies in the fact that this may take the place of CCS pressure measurement which requires a catheter to be inserted into the sinus, a technique which is difficult and prone to clogging despite the use of heparin.

Histologic Results. Histological assessments of SP-74 and SP-76 present variability in the placement of electrodes and demonstrated a similar profile of tissue changes that have been presented in previous QPRs. To avoid redundancy, we have not included figures of histology for these two animals. In SP-74, three of the four electrode tips (#2, 3 and 4) were positioned within the mediolateral or ventral white matter, while the tip of electrode #1 was seen within the left ventral gray horn. In SP-76, the tips of electrodes #1 and #2 were observed within either the right or left mediolateral white matter, while electrode #3 was seen at the margin between gray and white matter of the left ventral gray horn. Electrode #4 was positioned within the right ventral gray matter. The results of histological analysis of SP-75 are inconclusive due to the nature in which we divided the tissue. We did, however identify partial electrode tracks for two of the six electrodes but no tips were identified.

Other cellular changes observed within the tissue surrounding the electrode capsules in all animals included fibrosis associated with the electrode track. Noted were reactive astrocytes, stellate fibroblasts and associated collagen bundles, vascular hyperplasia, and myelin changes including Wallerian degeneration with nearby lipid-laden macrophages. Variably-sized inflammatory foci, usually associated with trauma to the pia and leptomeningeal vessels, was seen especially in SP-74 near the tip of electrodes #3 and #4, as well as in SP-76 near the tips of electrodes #1 and #2. Neurons in the vicinity of the foci of tissue injury appeared normal in all three cats.

An attempt to demonstrate horseradish peroxidase (injected intravenously prior to vascular perfusion) together with the localization of NADPH-d in SP-75 was unsuccessful. We have not yet attempted to analyze additional material in this animal for perioxidase localization at present. AP

activity noticeably labeled both the luminal and abluminal vascular surfaces of many small blood vessels of the dorsal and ventral cortex of the cervical spinal cord (Figs. 8-11). NADPH-d labeled neurons of the dorsal gray horns and their fibers extending through Lissauer's tract (Figs. 12-15). NOS labeling was observed in some of the neurons and fiber tracks within the dorsal gray and white matter of the cervical spinal cord (Figs. 16 and 17). NOS was also seen staining neurons lateral to the central canal, which is consistent with the literature. Whether or not NOS co-localizes with NADPH-d within the spinal cord has not been determined to present.

Discussion

Our finding here that a 12 hour period of continuous stimulation delivered on two successive days did not significantly alter neuronal excitability confirms our earlier results and this information will be added to our database. In review, a pattern has emerged suggesting that the neural elements within the spinal cord are quite resilient to high levels of electrical stimulation. Instead, the major component of injury appears to be the positional instability of the electrode array. We continue to explore design alternatives to minimize this mechanical injury.

Acute experiments aimed at eliciting sexual reflexes have proven challenging. When not filled with blood, the corpus cavernosum sinus (CCS) of the cat is very small, thin, and spongy making correct catheter placement difficult. Small catheters must be used which may become clogged despite the use of heparinized saline. The delay between stimulation onset and an increase in CCS pressure is typically more than 30 s and at least 3 min must be allowed between trials so as not to elicit spinal depression. This makes proper electrode positioning time consuming.

Microstimulation of the spinal cord induced an increase in the CCS pressure and a lengthening of the penis. We expected that there might be a relaxation of the EUS and an increase in pressure at the base of the bladder. Such changes would be useful in preventing retrograde ejaculation.

However, the intraurethral pressure profiles taken at different levels of CCS pressure did not differ remarkably other than the expected increase in penile urethral pressure.

The newly performed cytochemical and immunocytochemical experiments were essentially pilot studies performed to develop technical skills for future experimentation with cat CNS tissues. Unfortunately, we were unable to extract information concerning electrode placement in SP-75. However, this animal was important as we were able to work out some of the technical difficulties, albeit not complete at present, especially for the localization of AP, NOS, and NADPH-d within the spinal cord.

AP is an enzyme that is localized within the plasma membranes of cells from several tissue of the gut, liver, kidney, and bone. In the CNS, it has been established to play an important role with other phosphatase enzymes for blood-brain barrier (BBB) modulation. AP in the CNS is primarily localized within the endothelial cell (EC) plasma membranes, primarily on the luminal surface (blood-facing front) of the ECs. During injury to the CNS, there is a shift of the localization and expression of AP to the abluminal EC plasma membrane. Thus, this enzyme can be considered to be a useful marker of BBB injury (Vorbrodt, 1988), and will be a useful marker for future planned studies of BBB permeability in electrically stimulated animals.

As was already pointed out, various neuronal populations within the CNS exhibit NADPH-d activity. Because this enzyme purpotedly co-localizes with NOS, the enzyme responsible for the synthesis of the neurotransmitter nitric oxide (NO) in some of these neurons, based on biochemical studies it is thought that neuronal NADPH-d is identical to NOS and that NADPH-d histochemistry can serve as a specific marker for NO-containing neurons (Vizzard et al., 1994). Previous studies have suggested the possibility that NO may play a role in autonomic pathways, and NADPH-d activity has been identified within bladder and pelvic afferent neurons. Because previous studies have shown that damage to ventral roots in the rat upregulates NOS in motor neurons of the ventral

lumbar spinal cord (Wu, 1993), one may expect changes in the expression of these enzymes as a function of cell injury. The application of NADPH-d and NOS in animals subjected to electrical stimulation of the sacral spinal cord, therefore, may offer better understanding of the role that these enzymes play in spinal autonomic pathways in the cat.

Future Work

In the next quarter we plan to test a new chronic design featuring two, 3-microelectrode arrays arranged longitudinally. We also plan to conduct an experiment exclusively for gathering cytochemical and immunocytochemical data on tissues within the vicinity of the electrode tracks. Studies employing the above mentioned enzymes and antibodies developed against NOS and other neuronal, glial, or endothelial cell proteins will be useful to study potential changes that may occur in spinal cord neurons, glia, and vasculature as a function of electrical stimulation.

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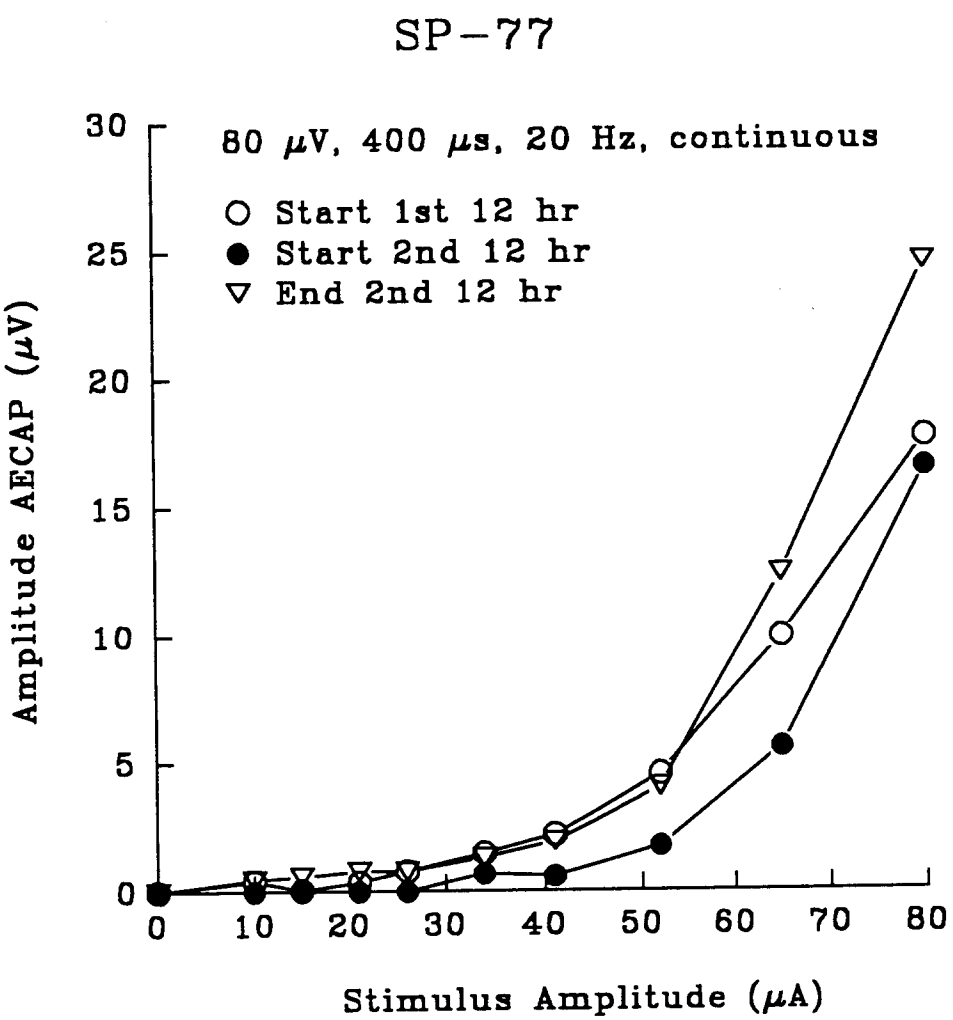

SP-77
80 μV, 400 μs, 20 Hz, continuous
Start 1st 12 hr
Start 2nd 12 hr
End 2nd 12 hr
Amplitude AECAP (μV)
30
25
20
15
10
5
0
0
10
20
30
40
50
60
70
80
Stimulus Amplitude (μA)

Figure 1

SP-79: Baseline Activity (10 Minutes)

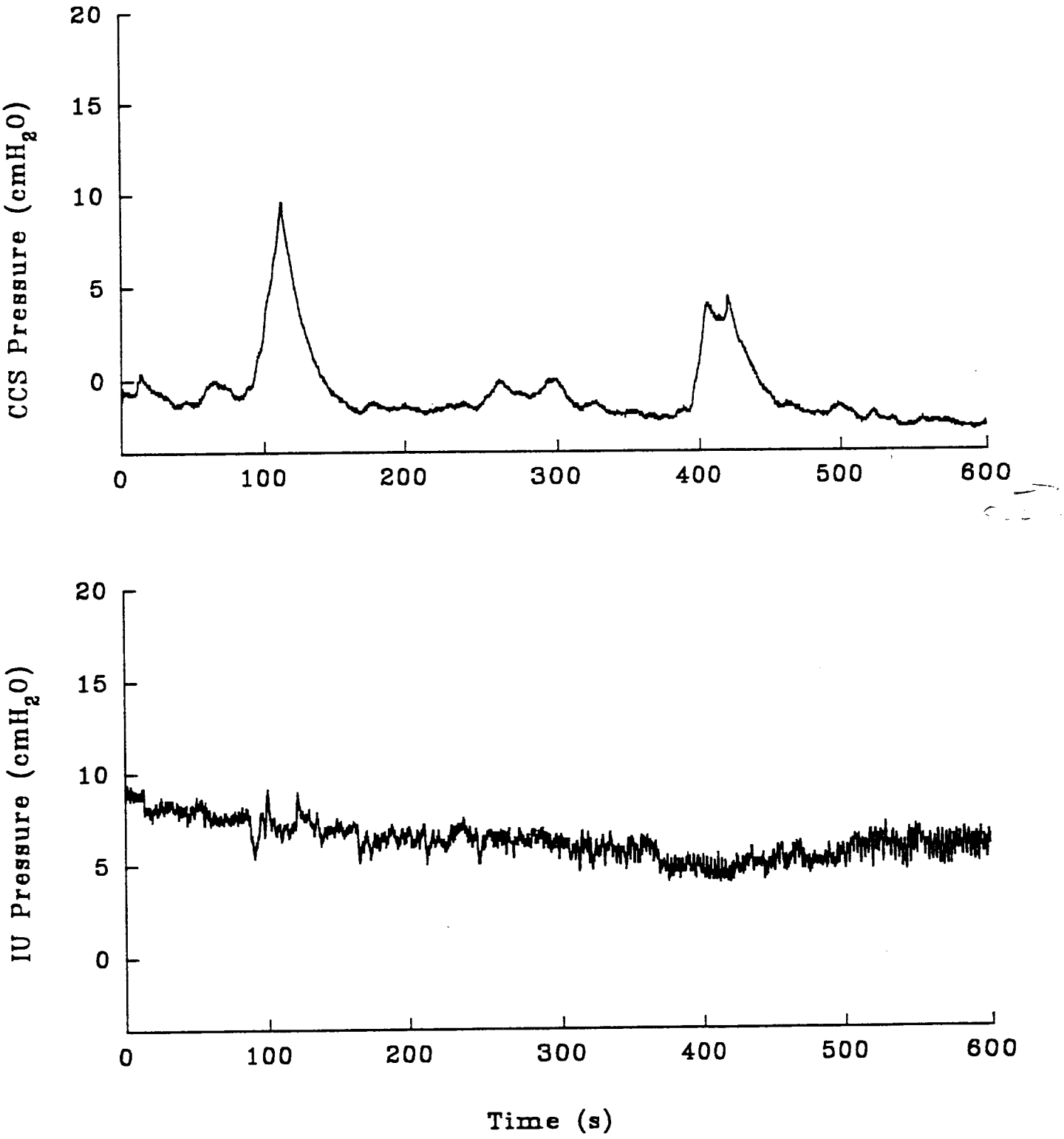


Figure 2

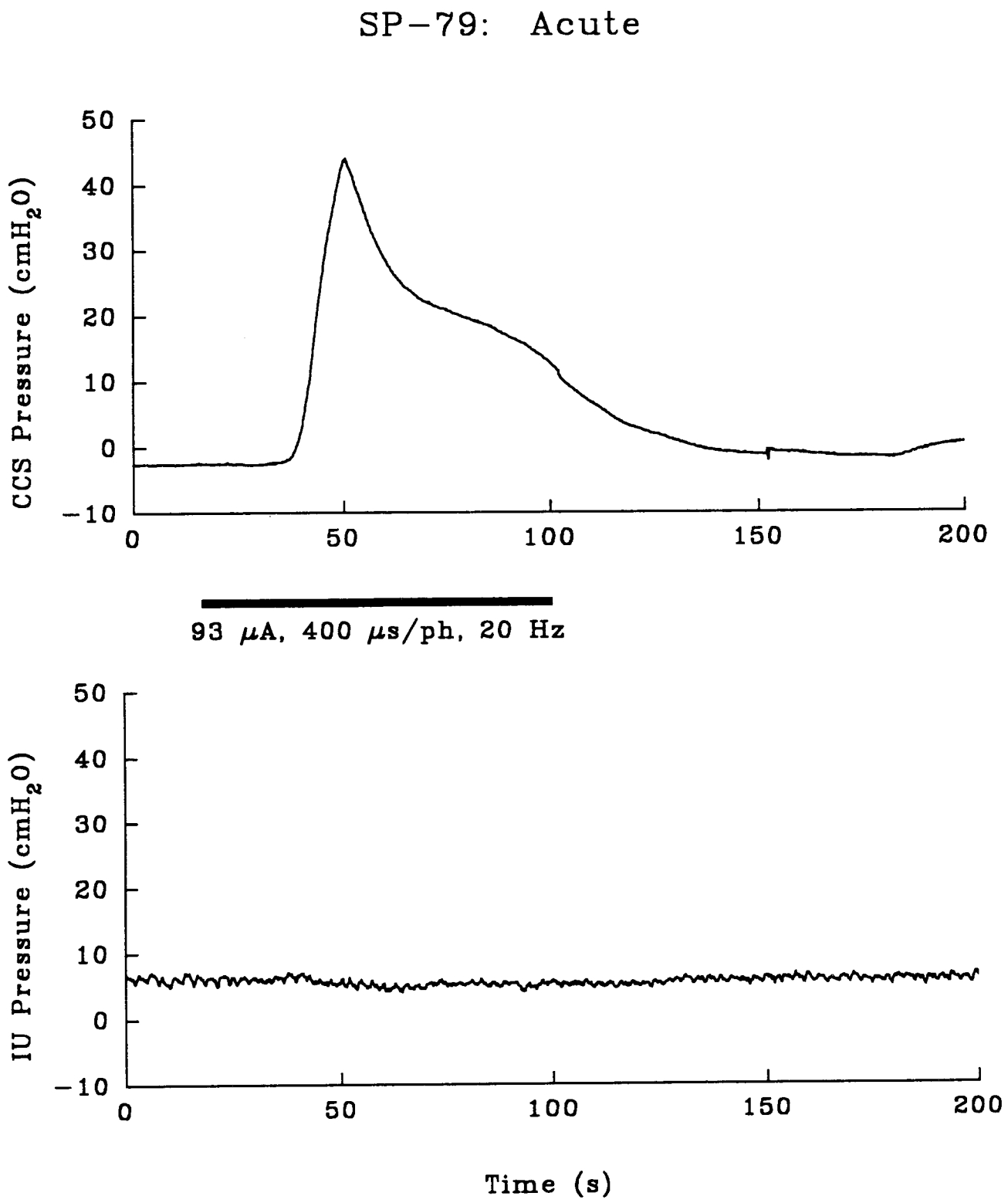

SP-79: Acute
CCS Pressure (cmH2O)
50
40
30
20
10
0
-10
0
50
100
150
200
93 μA, 400 μs/ph, 20 Hz
IU Pressure (cmH2O)
50
40
30
20
10
0
-10
0
50
100
150
200
Time (s)

Figure 3

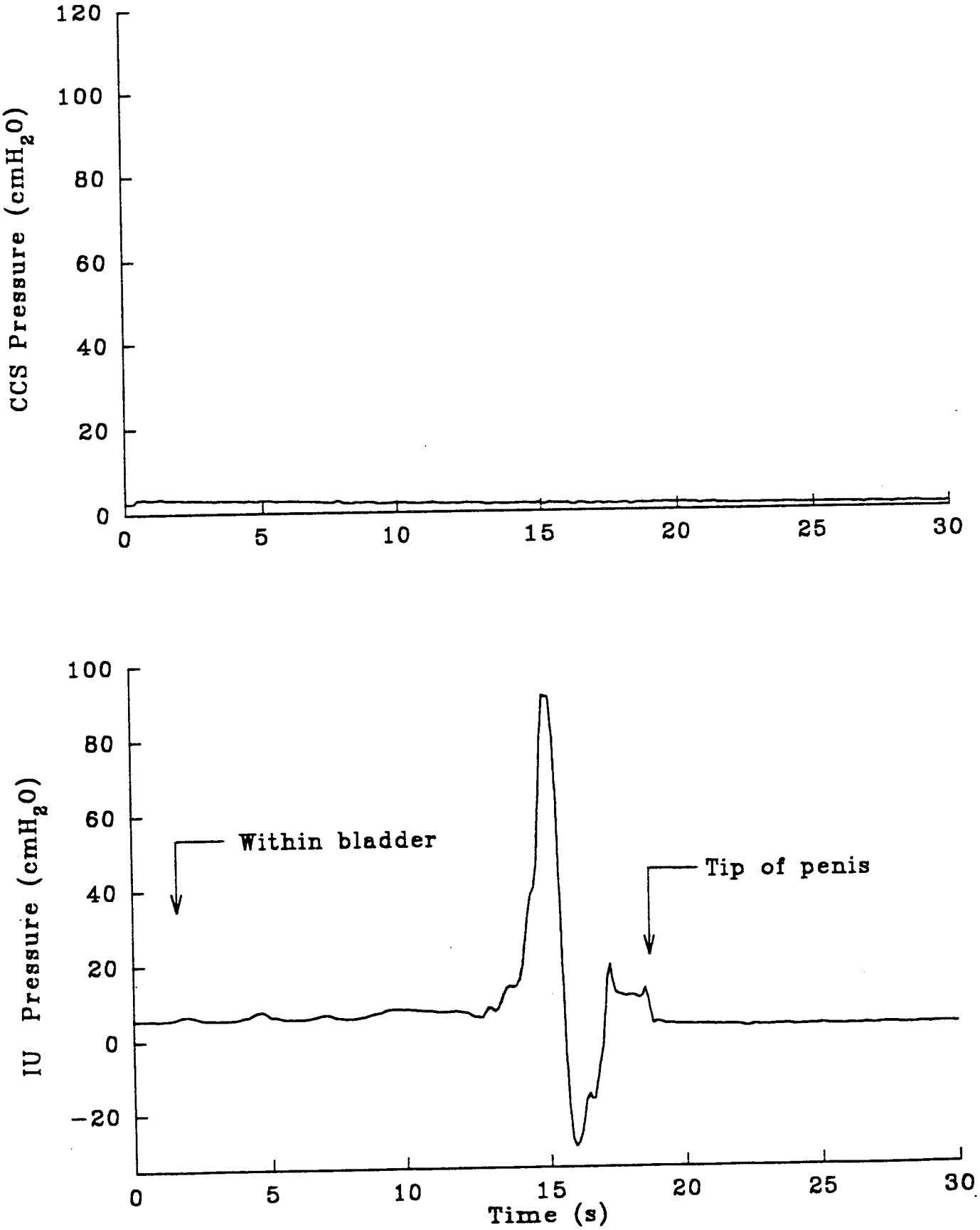

SP-79: Acute
CCS Pressure (cmH_2O)
120
100
80
60
40
20
0
0
5
10
15
20
25
30
IU Pressure (cmH_2O)
100
80
60
40
20
0
-20
Within bladder
Tip of penis
0
5
10
15
20
25
30
Time (s)

Figure 4

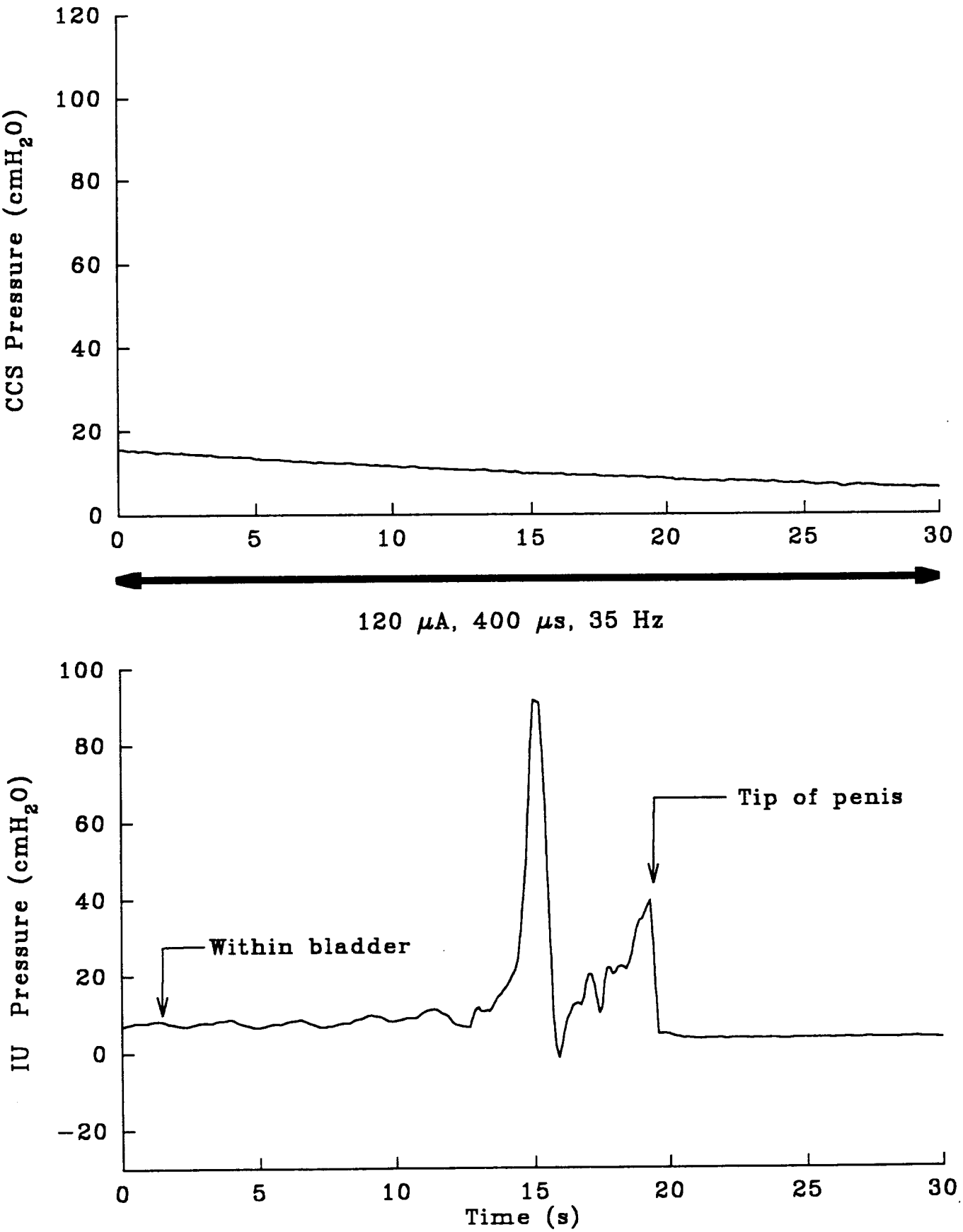

SP-79: Acute
CCS Pressure (cmH_2O)
120
100
80
60
40
20
0
0
5
10
15
20
25
30
120 μA, 400 μs, 35 Hz
IU Pressure (cmH_2O)
100
80
60
40
20
0
-20
Within bladder
Tip of penis
0
5
10
15
20
25
30
Time (s)

Figure 5

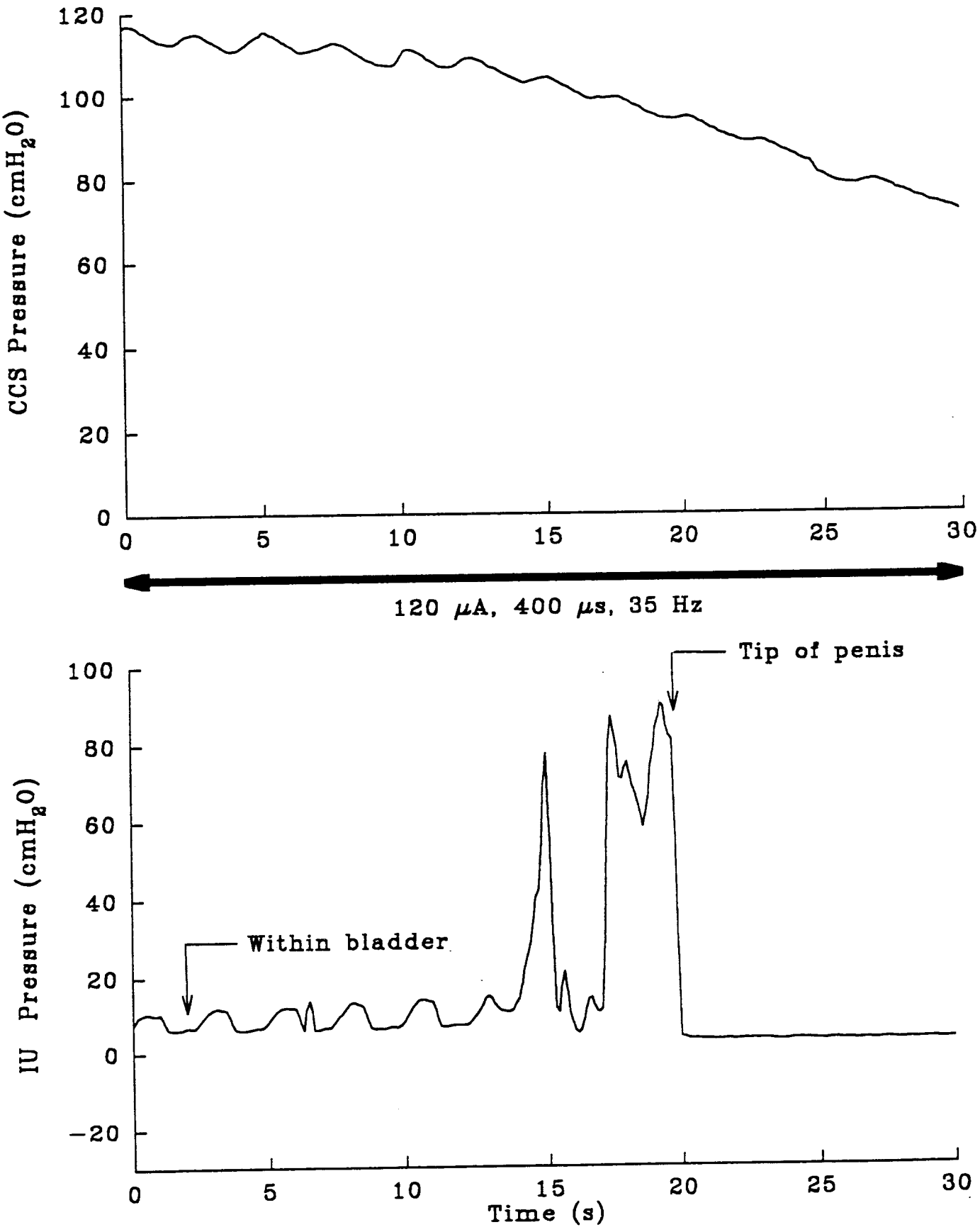

SP-79: Acute
CCS Pressure (cmH_2O)
120
100
80
60
40
20
0
0
5
10
15
20
25
30
120 μA, 400 μs, 35 Hz
IU Pressure (cmH_2O)
Tip of penis
Within bladder
100
80
60
40
20
0
-20
0
5
10
15
20
25
30
Time (s)

Figure 6

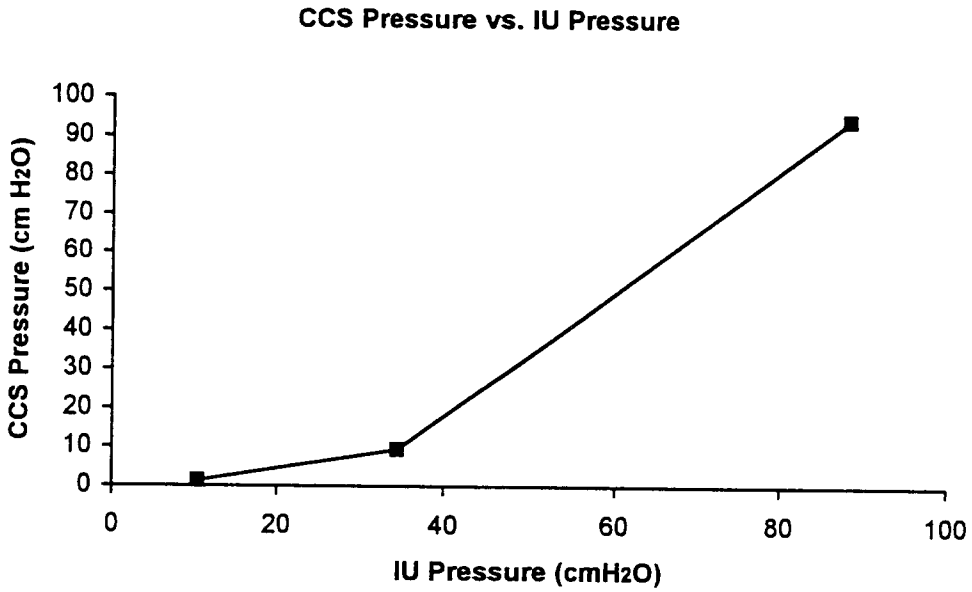
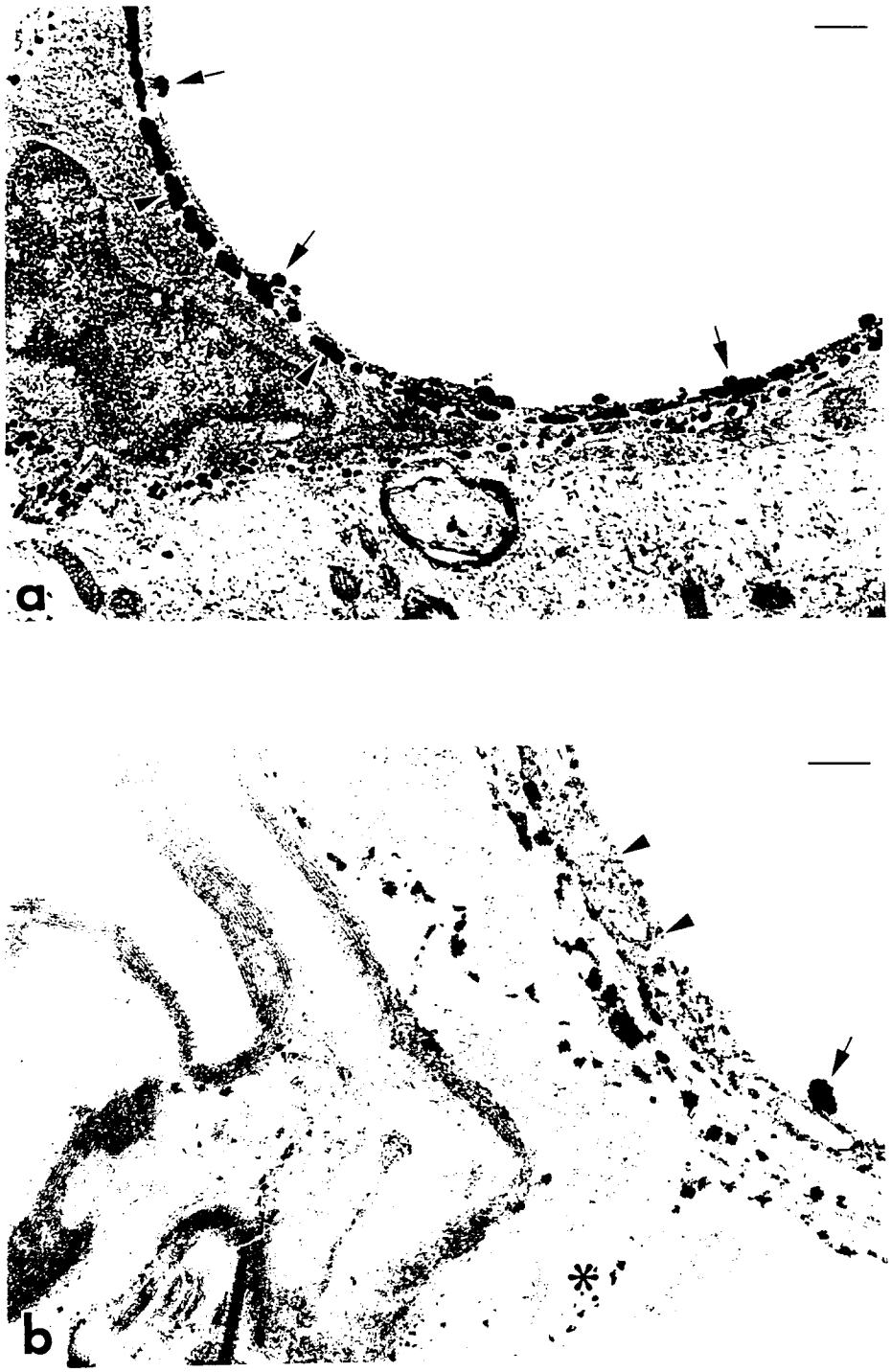
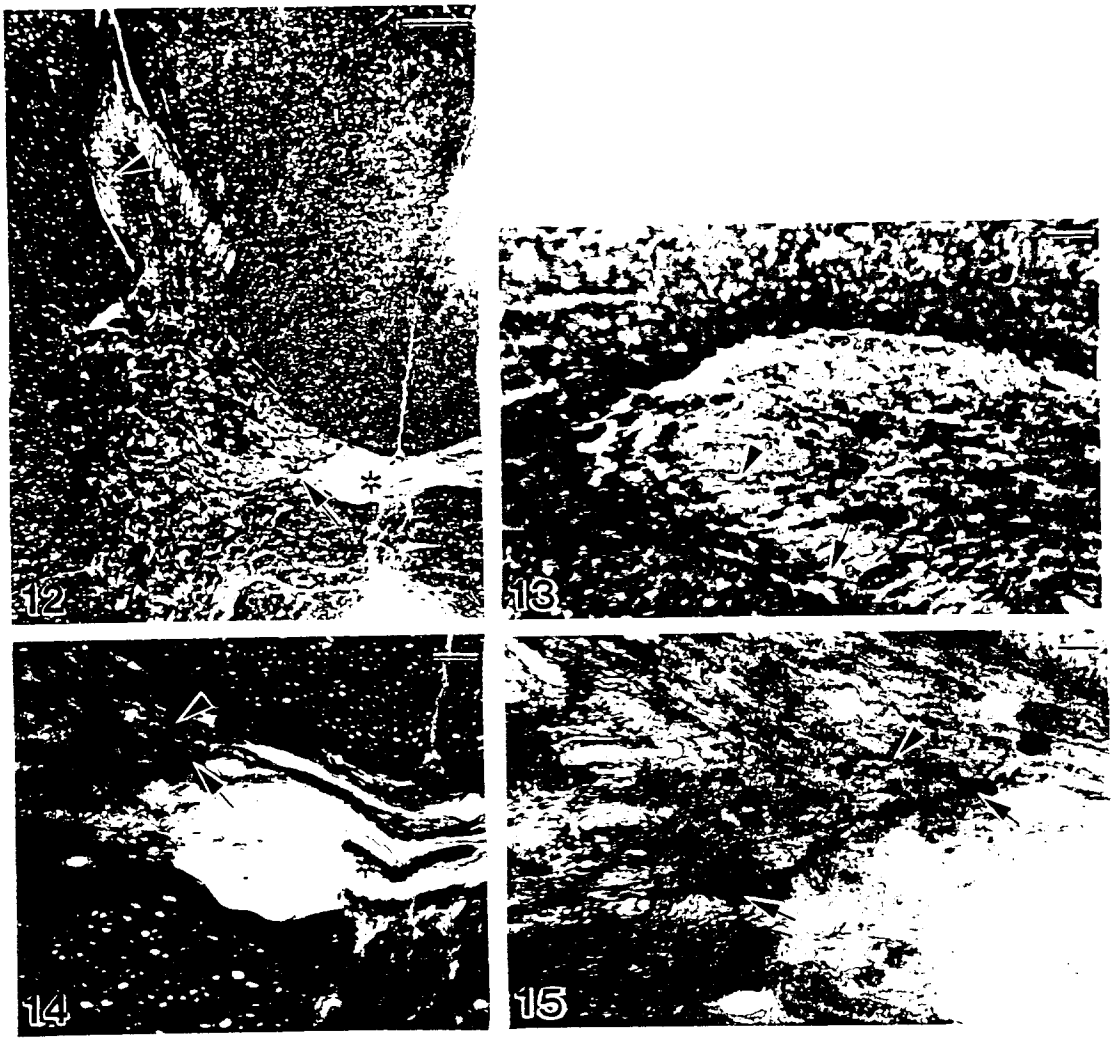

CCS Pressure vs. IU Pressure
CCS Pressure (cm H2O)
100
90
80
70
60
50
40
30
20
10
0
0
20
40
60
80
100
IU Pressure (cmH2O)

Figure 7

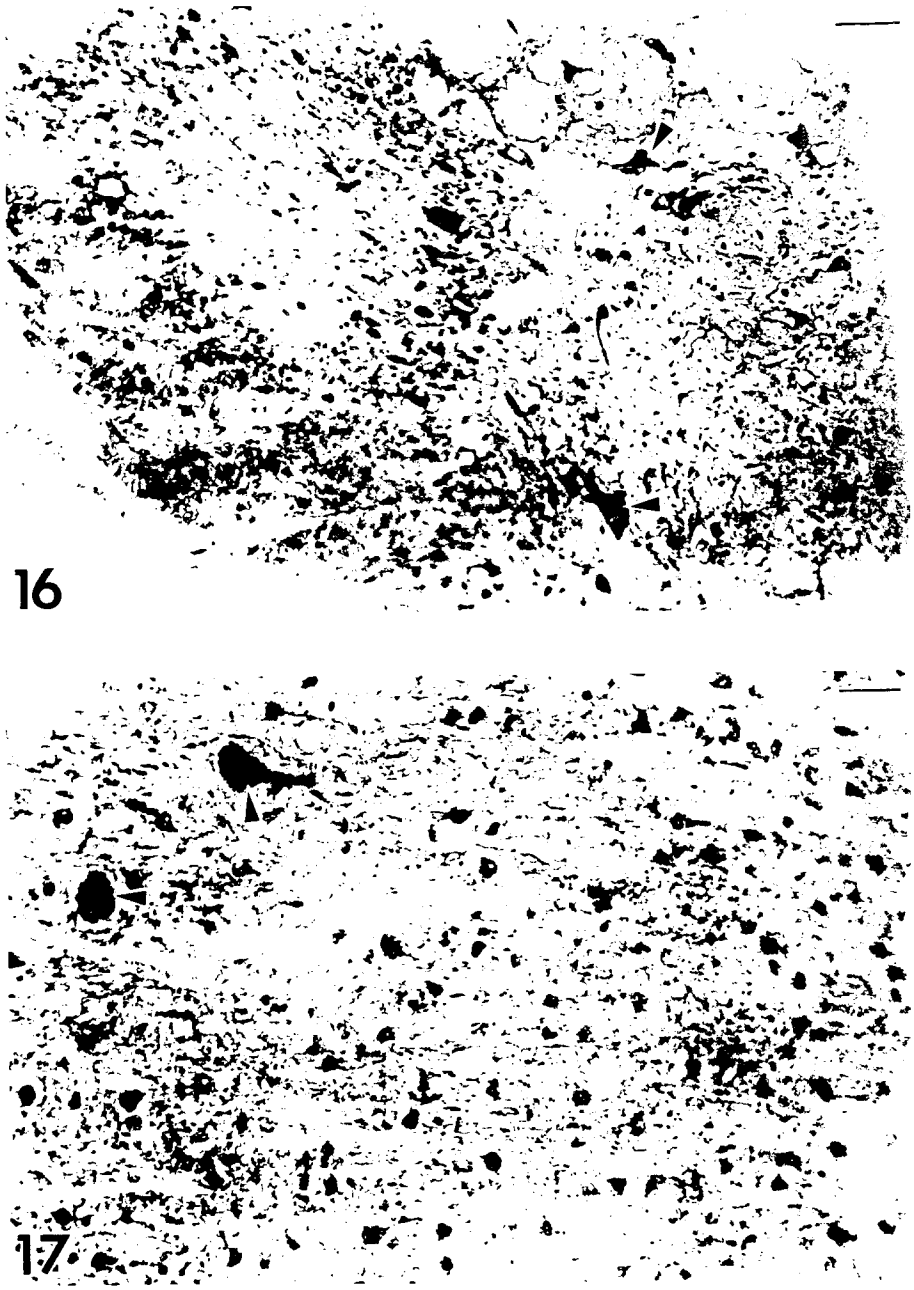
Figs. 8-10. Figures 8-10, 12-16 represent 50 μm-thick sections, SP-75, cervical spinal cord. These light micrographs represent the reaction product for alkaline phosphatase (AP) associated with small blood vessels of the gray and white matter. Note in Fig. 10 the stronger reaction product within capillaries and arterioles (arrows), while post-capillary venules and veins show a weaker reaction (arrowheads). Bars = 8: 250 μm; 9: 100 μm; 10: 50 μm.



Figs. 11a, b. a. These two figures are transmission electron micrographs from SP-75, cervical spinal cord. Note the black precipitate material characteristic of AP located on the luminal (arrows) and abluminal (arrowheads) endothelial surfaces. b. The reaction product for AP within the endothelial cells is shown more granular in this micrograph (arrowheads), some of which is shown within the extracellular space (*). Bar = a: 0.50 μm; b: 0.25 μm.



Figs. 12-15. Cervical spinal cord, SP-75. The cytochemical reaction for NADPH-d is shown associated with neurons and fiber tracks of the pericentral canal region (shown collapsed *) and fibers extending to the dorsal horn (arrowheads) (Figs. 12, 14, 15), and also within neurons (arrows) and fibers within Lissauer's tract of the dorsal horn (Fig. 13). Bars = 12: 250 μm; 13: 50 μm; 14: 50 μm; 15: 25 μm.



Figs. 16, 17. Cervical spinal cord, SP-75. NOS immunocytochemical reaction is shown staining small and large neurons of the dorsal gray horn (arrowheads). Bars = 16: 50 μm; 17: 50 μm.